REACTIVITIES OF THE CYSTEINYL RESIDUES OF HUMAN CERULOPLASMIN (FERROXIDASE)

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1. Introduction

Ceruloplasmin (ferroxidase) is one of the very few oxidases capable of catalyzing the reduction of molecular oxygen to water. With the exception of cytochrom oxidase these enzymes all contain four or more copper ions, are intensely blue, and apparently work according to identical or very similar mechanisms [1]. Cysteine has repeatedly been suggested to participate in copper binding and enzymatic reactions. Three different kinds of copper and electron acceptors have been identified. One of these is a two-electron acceptor, which is not seen in electron paramagnetic resonance [2]. The absence of a signal was first attributed to spin pairing of two bivalent copper ions [3]. An alternative model suggested by Byers et al. [4] involves the binding of two copper ions by a disulphide bridge, which would then be reduced to a pair of thiol groups upon accepting the two electrons.

Cysteine has also been implicated in the binding of the blue (type1) copper. This type of copper is present in a number of small blue proteins such as the azurins from bacteria and the plastocyanins from green plants. Amino acid sequence studies [5,6] show that all of these proteins contain a single cysteine residue in a region of the peptide chain that has been highly conserved during evolution, suggesting a functional role for the thiol group. Direct evidence for the involvement of cysteine in copper binding has been obtained by Morpurgo et al. [7].

Abbreviations:
CM-cysteine : *S*-carboxymethyl-cysteine;
EDTA : Ethylene diaminetetraacetic acid, sodium salt;
Tris : Tris(hydroxymethyl)amino methane.

We have undertaken a study of the cysteine residues in ceruloplasmin preliminary to primary structure determinations. Previous work [8,9] provided no information regarding a possible change in the number of thiols in the enzyme upon its reduction by reducing substrate, nor was the number of such groups in the resting enzyme unequivocally determined. By using alkylation with radioactive iodoacetic acid we have measured sulphydryl groups in ceruloplasmin in resting and ascorbate reduced form, under native as well as dissociating conditions. The data which show that 3 groups are present in both forms of the enzyme, allow us to refute the model of Byers et al. [4] for the two-electron acceptor. They also show that none of the cysteines are accessible to iodoacetic acid in the native enzyme

2. Experimental

2.1. *Material*

Human ceruloplasmin was prepared from fresh retroplacental serum as described earlier [10]. The alkylating solution used throughout was iodo-2-[C^{14}]-acetic acid (The Radiochemical Centre, Amersham) diluted with cold sodium iodoacetate (Sigma) in water to get a final concentration of 24.2 mM and a specific activity of 0.239 μCi/μmol as determined by iodine analysis and scintillation counting using internal standardization. The guanidine hydrochloride was Serva p.a. (A_{260} less than 0.01).

2.2. *Alkylation*

5–10 mg of ceruloplasmin in 0.5 ml of 0.7 M Tris–HCl, pH 8.6, containing 5 mM EDTA was incubated

with 0.1 ml of the alkylating solution, corresponding to an approx. 50-fold molar excess over enzyme (15-fold molar excess over SH-groups). After 1 hr at room temperature the protein was separated from the reagents on a Sephadex G-50 column (1.5 × 32 cm) in 0.05 M ammonium acetate, pH 8.6.

Reduced enzyme was obtained by flushing the ceruloplasmin solution with nitrogen and then adding a 20-fold molar excess of ascorbic acid (3-fold molar excess over enzyme copper) in water, which caused instantaneous decolourization.

Alkylation of oxidized (resting) denatured enzyme was effected by adding solid guanidine hydrochloride to the incubation mixture to get a concentration of 50% (w/w) (6 M).

Alkylation of ascorbate-reduced enzyme under native conditions was effected by adding 0.1 ml of the alkylation solution to the reduced enzyme. Alkylation of ascorbate-reduced enzyme under denaturing conditions was done by treating the reduced enzyme with 1 ml of saturated guanidine hydrochloride in buffer containing 0.2 ml of the alkylating solution. The latter mixture was thoroughly flushed with nitrogen before addition to the enzyme solution, which was also kept under nitrogen throughout.

2.3. *Analysis*

The amino acid analyses were done with an updated Beckman Model 120 amino acid analyzer and a Nuclear Chicago No. 6352 bench top liquid flow scintillation counter system equipped with a 2 ml Packard Kel F flow cell, No. 7600–108 packed with anthracene. The specific activity of the CM-cysteine obtained in the alkylation experiments, expressed as observed counts per nmol, was determined in the above system as follows. A 20-fold molar of cysteine in water adjusted to pH 8 with NaOH was alkylated with the iodoacetic acid solution. An amount of the reaction mixture containing about 100 nmol of CM-cystein was then analyzed. The ninhydrin colour value used for the CM-cysteine was obtained by running a weighed standard of the amino acid with the scintillation flow cell included in the system. The value obtained was 270 counts per nmol of CM-cysteine. This permitted a counting accuracy of 2.5% at a 95% level of confidence for the labelled protein samples.

1 mg of protein sample was lyophilized and hydrolyzed in the presence of 1 μmol of cold CM-cysteine in 6 M HCl at 110°C for 24 hr in sealed evacuated tubes. The recovery of CM-cysteine in the hydrolysis, as determined by standard addition using the labelled CM-cystein obtained in the alkylation experiment described above, was 95.4%. When large excess cold CM-cysteine was not included in the hydrolysates the recovery fell dramatically. The low recovery of CM-cysteine in acid hydrolysates of ceruloplasmin, combined with interference by methionine sulfoxide with chromatographic determination, necessitated the use of the indirect approach described herein for CM-cysteine analysis. The amino acid analysis data were normalized by taking the sum of Gly, Ala, Val, Ile, Leu, Tyr and Phe to be 415 residues. This is consistent with complete compositional data for the protein assuming that it contains a total of 1065 amino acid residues [11], corresponding to a mol. wt of 134 000.

3. Results and discussion

The results of the alkylation experiments are reported in table 1. No radioactivity was incorporated in the native enzyme. A small amount of radioactivity was incorporated by an older preparation which had lost some of its blue colour. This might explain the earlier results [8,9] which indicated that a single cysteine residue was accessible in the native enzyme.

After denaturation with guanidine hydrochloride, 3 cysteine residues are reactive. The precision of the determination is estimated at 3–5%. The alkylation should be complete under the conditions used. Calculation of absolute specific activities or counting efficiencies do not enter since the same alkylating solution was used throughout and the counting in the flow cell is always done in the same medium and at the same flowrate. The determination should therefore have the same error as normal amino acid analysis, which generally is 3% or better in this laboratory. The only additional source of error is counting statistics. The determination thus affords a new independent measure of the mol. wt, the result of which is 3 residues of cysteine per 134 000, in excellent agreement with earlier data [11].

The total number of half-cystein plus cystein residues in ceruloplasmin was found to be 15 (unpublished data). We then conclude that the protein contain 6 disulphide bridges. Two type 1 copper ions are present

Table 1
Analysis of *S*-carboxymethyl-cysteine in samples of ceruloplasmin alkylated by iodoacetic acid under different conditions

Buffer	Moles of CM-cysteine per mole of protein	
	Oxidized (resting) form of enzyme	Ascorbate-reduced form of enzyme
0.7 M Tris–HCl, pH 8.6 5 mM EDTA	0.00	0.00
0.7 M Tris–HCl, pH 8.6 5 mM EDTA, 5–6 M guanidine hydrochloride	2.98	3.32

in ceruloplasmin [12]. The number and accessibility of the thiol groups in the protein does therefore not exclude the possibility that they are involved in binding this copper.

In the experiments with ascorbate-reduced enzyme we found that iodoacetic acid was a powerful inhibitor of ceruloplasmin. No loss of blue colour occured if the iodoacetate was added before the ascorbate solution. The iodoacetate is possibly included in the group of inhibiting carboxylic acids studied in detail by Gunnarsson and Pettersson [13].

The number of thiol groups is not different in the ascorbate-reduced as compared to the resting enzyme. The small but significant increase seen might reflect a slow reduction of disulphides by the dehydroascorbate produced in the reduction of the enzyme. The results allow us to refute the idea of Byers et al. [4] that a disulphide bridge functions as a two-electron acceptor in the enzyme. The most likely model for this acceptor seems then to be a pair of spin-coupled cupric ions, as suggested by Fee et al. [3]. A third possibility, which has been advocated by Hamilton et al. [14] for galactose oxidase, is a trivalent copper ion. This model seems unlikely, since the Cu^{3+} ion is very unstable and is expected to oxidize most ligands in proteins [15].

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